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Immunoblotting for the serodiagnosis of alveolar echinococcosis in alive and dead Eurasian beavers (*Castor fiber*)

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Abstract

A novel species-specific anti-beaver-IgG-alkaline-phosphatase conjugate was synthesized for the development a of a new serological test for echinococcosis in beavers. Two different ELISAs conventionally used for human *Echinococcus multilocularis* serology (Em18-ELISA and Em2-ELISA) yielded diagnostic sensitivities of 0% and 46%, respectively. In contrast, the subsequently developed immunoblotting assay gave an 85% diagnostic sensitivity (11 out of 13 beavers with alveolar echinococcosis were immunoblotting-positive, i.e. showed reactivity with a specific 21 Mr band), and maximal specificity. In conclusion, this immunoblotting assay should be the method of choice for use in serological studies on *E. multilocularis* in Eurasian beavers, and the test proved suitable to investigate both animals alive and post-mortem.

Keywords

Echinococcus multilocularis; beaver; Em2-ELISA; Em18-ELISA; immunoblotting; EmVF-antigen

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Introduction

Echinococcus multilocularis is one of the most pathogenic parasitic zoonoses prevalent in central Europe. The definitive (final) wildlife hosts in Europe are canids, including predominantly the red fox (*Vulpes vulpes*), but the non-native raccoon dog (*Nyctereutes procyonoides*) and the domestic dog are affected as well (Carmena and Cardona, 2013; Carmena and Cardona, 2014). Adult tapeworms live in the small intestine of the definitive hosts, from which gravid parasite proglottids and eggs are shed with the faeces into the environment. Intermediate hosts are infected when they ingest parasite eggs, which upon release of an oncosphere, develop into the larval stage called metacestode. Metacestodes show a distinct predilection for the liver. At a later stage of infection, metastases develop predominantly in the lungs and brain, though other organs can be affected. The metacestode proliferates continuously and leads to a cancer-like disease in affected intermediate hosts. The sylvatic cycle is completed by carnivore predation of such infected intermediate hosts. In Europe several species of small microtine and arvicolid rodents are the principle intermediate hosts, as well as two larger introduced species, the coypu (*Myocastor coypus*) and the muskrat (*Ondatra zibethicus*) (Mathy et al., 2009). The Eurasian beaver (*Castor fiber*) is another intermediate host. The first cases were reported from Switzerland (Janovsky et al., 2001; Wimmershoff et al., 2012) and Austria (Cronstedt-Fell et al., 2010), and more recent cases were described in the United Kingdom (Barlow et al., 2011) and in Serbia (Cirovic et al., 2012). Humans are paratenic ("accidental") intermediate hosts.

A beaver reintroduction to Britain is currently underway as a scientific trial in Scotland, with a large population of free-living beavers now established on the east coast. Further releases have been proposed in Wales, and feasibility investigations are undertaken in England. However, there is now another growing population of free-living beavers, which have either escaped from captivity or been purposely, released in Scotland and England but are not part of government-sanctioned trials. The origin of beavers for importation has been subject to academic debate (Halley 2011, Rosell et al. 2012), the health status of imported animals being a key parameter. Current prevention measures include a six months quarantine period, which is deemed sufficient to prevent the associated entry of rabies. Screening for other infectious organisms is not required during this period, but additional health screening recommendations have been made (Goodman et al. 2012). Among others, the risk of introduction of *E. multilocularis* to non-endemic regions via importation of beavers originating from endemic areas has been assessed following the Office International des Epizooties (OIE) risk assessment framework (Kosmider et al., 2013; Defra, 2012). Subsequently, cases have been detected among beavers meant for reintroductions (Cirovic et al., 2012; Barlow et al., 2011), and it is now recognized that both captive and wild-caught beavers from central Europe represent a risk to import *E. multilocularis* to presently *E. multilocularis*-free areas (Barlow et al. 2011; Campbell-Palmer et al., 2012; Pizzi et al., 2012).

So far, cases of echinococcosis in beavers have mainly been diagnosed by post-mortem investigation, principally based on methods that have been widely used to identify the larval stage of *E. multilocularis* in other rodents and in human patients. Beside conventional histopathology, a molecular analysis by PCR and/or direct immunofluorescence yields a reliable species-specific identification of the parasite. This is particularly helpful in the rare cases where histology findings are inconclusive (Diebold-Berger et al., 1997).

In contrast, diagnosing alveolar echinococcosis in live beavers is challenging. Imaging procedures may provide a certain degree of information, as it has been demonstrated in other rarely infected intermediate hosts, e.g. dogs (Scharf et al., 2004), rats (Asanuma et al., 2005) and non-human primates (Kishimoto et al., 2009) but it is not reliable enough to rule out an infection. Currently, investigation of live beavers prior translocation includes a time-consuming combination of clinical examination and diagnostic imaging, such as detailed abdominal ultrasonography combined with endoscopic surgical visual examination of the liver and other abdominal organs in anaesthetized animals (Pizzi et al., 2012). A serological test could be used as a rapid diagnostic tool that could considerably reduce such investigations in beavers. Besides its application to prevent the entry of the parasite via imported individuals, serology could also be useful to assess exposure in captive or free-living populations considered potential sources for translocation projects as well as to estimate prevalence in infected populations. To our knowledge, serological diagnosis of parasitic infections in beavers has not yet been reported. The goal of this study was to elaborate and evaluate serological tests regarding their suitability to diagnose an *E. multilocularis* infection in the Eurasian beaver.

Materials and Methods

Study design, animals and samples

We comparatively evaluated several conventional antigens presently used to detect anti-*E. multilocularis* antibodies in intermediate hosts such as humans and small rodents (crude vesicle fluid, EmVF-antigen [Müller et al., 2007], Em2-antigen [Gottstein et al., 1991], Em18-antigen [Sako et al., 2002).

In a first step we developed a new anti-beaver-IgG-specific secondary antibody, because we had found out in preliminary experiments that heterologous conjugates (anti-mouse IgG; protein A, protein G) yielded unsatisfactory results with beaver samples. This host-specific antibody, at an affinity-purified status, was coupled to alkaline phosphatase by using a conventional procedure provided by the manufacturer (Sigma-Aldrich, http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/alkaline-phosphatase/conjugation.html).

In a second step, we compared two ELISAs and immunoblotting as previously evaluated for human alveolar echinococcosis serology (Müller et al., 2007). Test evaluation was done using the samples from three different groups of beavers: (1) a "positive" group of 13 beavers confirmed to be infected with *E. multilocularis* by post-mortem investigations including histopathology and PCR (nine animals of Swiss origin and four animals of Austrian origin); (2) a "negative" group consisting of 27 beavers originating from a region known to be non-endemic for *E. multilocularis* (Scotland), i.e., animals expected to have not been exposed to the parasite; (3) another "negative" group including 29 beavers from areas endemic for *E. multilocularis* (25 dead animals from Switzerland and four from Austria), i.e., with possible previous exposure. All negative beavers (groups 2 and 3) were necropsied and did not present lesions consistent with the presence *E. multilocularis* infection. For statistical determination of a negative-positive-threshold value in ELISA, only negative animals from group (2) were used.

For all beavers, blood samples consisted in sero-sanguinous fluid collected post-mortem, either obtained from clotted heart-blood, or (if not available) muscle juice obtained as described elsewhere (Berger-Schoch et al., 2011). Samples were kept frozen at -20°C until further analysis.

Anti-beaver IgG-conjugate

One ml of frozen beaver blood was used to purify IgG with the ammonium sulfate precipitation technique as described by Page and Thorpe (2002), by applying a three-cycle-precipitation at 40%, 40% and 50% saturation conditions. Purified beaver IgG was sent to Gallus Immunotech Inc. (Ontario, Canada) for the production of affinity-purified chicken anti-beaver-IgG, based on IgY isolation from eggs derived from hens immunized with 100µg beaver-IgG emulsified in Freund's adjuvants (indications refer

to one immunization shot; hens received 1 primary immunization and 3 boosters every 10 days). Egg yolk IgY was subsequently immuno-affinity purified on a solid-phase beaver-IgG column as previously described (Felleisen and Gottstein, 1993). All subsequent steps to prepare the final anti-beaver-IgG-alkaline phosphatase-conjugate were carried out as described elsewhere (Baumann and Gottstein, 1987).

For a primary validation of the newly synthesized anti-beaver-IgG-conjugate, we electrophoretically separated the purified beaver-IgG by SDS-PAGE and applied a Coomassie-blue staining, followed by immunoblotting.

ELISA

All blood samples were examined for antibodies directed against the Em2- and recEm18-antigen from *E. multilocularis* by ELISA as previously published for foxes (Gottstein et al., 1991) but using the beaver-IgG-specific alkaline phosphatase conjugate described above. Beaver samples were considered sero-positive when the ELISA A405nm-values exceeded the average negative control value plus 4 standard errors (S.E.). The actual threshold value discrimination between negative and positive reactions was based on a 99.9% range exhibited by the 26 “negative” beaver samples from non-endemic areas, this was performed for each antigen separately. We used the mean plus 4 S.E. to calculate the threshold values for the Em2- and the recEm18-antigen, respectively. All values above these cut-offs were regarded as positive, all others below as negative. A ROC based approach was not feasible due to the low number of ”positive” cases.

Immunoblotting

Immunoblotting was performed as previously described for *E. granulosus* hydatid fluid (EgHF) antigen (Poretti et al. 1999), with the exception that EmVF (7 µg per cm slot) was used instead of EgHF (Müller et al., 2007). The conjugate was identical to that used for the ELISAs described above.

Statistical analyses

Data were analyzed using the computer program SPSS 17.0. One-way ANOVA and Student’s t-test were used to determine threshold values and to compare differences between groups. $P<0.05$ was considered as indicating statistical significance.

Results

Anti-beaver IgG-conjugate

Electrophoresis (Figure 1A) and Coomassie blue staining revealed the presence of two predominating bands, the upper one corresponding by relative molecular mass (Mr) to the heavy chain of the antibody, and the lower one to the light chain. Immunoblotting analysis of these two bands upon use of the anti-beaver-IgG-alkaline-phosphatase conjugate revealed the binding capacity of the conjugate to both antibody chains, with a stronger activity to the heavy chain (Figure 1B).

ELISA

Table 1 shows the results obtained with the two ELISAs for the beaver samples from the "positive" group. With the Em18-ELISA, all samples from this group yielded negative findings. With the Em2-ELISA, six out of 13 "AE-positive" beavers showed a serological reaction. Thus, the diagnostic sensitivities of both tests were very low (0% and 46%, respectively).

All 29 beaver samples of the "negative" group from endemic areas were serologically negative, i.e., the obtained values were all in the same range as the 27 "negative" samples from non-endemic areas used to determine the cut-off point. Statistically, there was no difference between the median value of the negative sera from non-endemic areas and those from endemic areas.

Immunoblotting

Immunoblot profiles, as shown for two samples from the "positive" group (beavers B1 and B2, Figure 2), demonstrated antibody reactivity with one major immunoreactive band and two minor side bands of approximately Mr 21 (major band), and Mr 19 and Mr 40 (minor bands). The localization of these three bands corresponded to the localization of bands obtained with a positive control of human origin (H1). This human serum banding pattern also matched the one described earlier in a large human serological study (Müller et al. 2007). Based on the detection of an anti-21Mr-banding activity, the immunoblotting approach yielded an 85% diagnostic sensitivity, as 11 out of 13 beavers from the "positive" group were seropositive, (Table 1). All "negative" and "true negative" beaver samples were clearly seronegative, considering the absence of any band (exemplified by samples B3 – B5 in Figure 2).

Discussion

The purpose of this study was to develop and evaluate the suitability of serological tests for the detection of *E. multilocularis* infection in the Eurasian beaver. Such a rapid diagnostic tool is urgently needed to facilitate the procedures aiming at minimizing the risk of introducing the parasite via translocated animals, which requires both the testing of translocated individuals in vivo prior to release, and screenings of potential source populations.

A serological test would be applicable on blood samples from both live and dead beavers, making it suitable for in-vivo testing of animals prior translocation and for serological surveys using samples collected post-mortem. The beaver-specific conjugate developed in this study operates methodically very well in ELISAs and also in immunoblotting assays, but the diagnostic performances turned out very different between the two test systems.

None of the beavers from the "positive" group showed a seropositive reaction in the Em18-ELISA, indicating that beavers do not develop a humoral immunity against this antigen. Results obtained with the Em2-ELISA were very unsatisfying a well. Thus, the evaluation of these two tests revealed that they are not suitable for diagnosis of *E. multilocularis* infections in beavers. One possible explanation for this phenomenon may not be related to the antigen itself, but may be due to the quality of the beaver "blood" used for serology. Post-mortem decay and degradation may decrease the serological quality of the fluids recovered from the dead animals with regard to application in ELISAs that use highly purified antigens, but may be not with regards to immunoblotting that uses the complex mixture of a crude metabolic antigen. We plan to investigate this aspect upon direct comparison of the Em2-ELISA and EmVF-IB with sera obtained from beavers captivated for translocation. However, such a study may be very lengthy in time, as we would need to investigate their livers post-mortem to get a conclusive diagnosis regarding the presence or absence of AE-lesions and *E. multilocularis* infection, respectively.

In contrast, a diagnostic sensitivity of 85% was observed with immunoblotting, which, in terms of serodiagnosis, reaches an acceptable level, especially as specificity reached 100% in our study. Overall, while a seropositive result in immunoblotting unambiguously indicates an infection (high positive predictive value), a negative serological result has to be considered with caution.

Interestingly, the tested seropositive beavers showed a very weak banding pattern in immunoblots. While a distinct and rather complex pattern of antigen bands is identified in samples of most human patients with alveolar echinococcosis, beavers exhibit a binding activity with a maximum of three different antigens. This suggests that the *E. multilocularis* metacestode antigens are of very weak antigenicity, which may partially explain why all beavers demonstrated negative results with the conventional Em18-antigen, and why only a very weak diagnostic sensitivity was obtained with the

conventional Em2-antigen. As discussed for the ELISAs above, one of the reasons why a few samples from infected beavers were negative in immunoblotting may have been a decreased quality of the beaver blood due to post-mortem decay. Another reason for the overall weak (methodically and diagnostically) humoral immune response detected in beavers with alveolar echinococcosis may be associated to a high susceptibility to infection of this animal species, i.e., infection and resulting organ lesions may occur in a way that the host cannot mount an appropriate humoral immune response, as compared to other intermediate hosts such as humans who react strongly by the humoral pathway of immunity. Nevertheless, this explanation is unlikely as we know from observations in laboratory rodents that antibody-deficient animals such as the µMT mouse do not show an increased susceptibility (Dai et al., 2004). However, a weak humoral immune response may be associated to a weak cellular immune response, and it is known from murine and human alveolar echinococcosis in immunosuppressed individuals that a weak cellular immune response markedly favours metacestode proliferation (Vuitton and Gottstein, 2010). Referring to beavers, this weak immune response is likely not a particularity of some putatively immunosuppressed individuals but rather a characteristic of the species itself. Indeed, if some beavers would develop a strong antibody reaction (without subsequent lesions), it is probable that part of the beavers from the “negative” group (i.e., animals without lesions but from endemic areas) would have been seropositive. Yet, our sample size was limited and serological investigations of a larger number of beavers originating from endemic areas and submitted to necropsy are necessary to further address this question. Furthermore, as in alveolar echinococcosis susceptibility to disease is usually associated to metacestode fertility, we invite wildlife pathologists to document the frequency of related findings, e.g. protoscolex formation within the parasite tissue, and to carefully record the features of periparasitic inflammatory and immune-mediated processes that may contribute to either accelerated or delayed metacestode proliferation and maturation (Vuitton and Gottstein, 2010).

Conclusions

Serodiagnosis of *E. multilocularis* infection in beavers is now possible, and so far the best methodical approach consists in performing immunoblotting based on the detection of anti-Mr21-band-binding activity. Cross- or non-specific reactions did not occur in our study (100% specificity), and the diagnostic sensitivity amounts to 85%. With these diagnostic sensitivity and specificity and a putative prevalence estimated in a future study area, positive and negative predictive values could now be determined. We encourage wildlife health scientists to make use of this tool in order to support further assessment of factors that will help to better interpret serological results.

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318

Table 1: Serological investigation of 13 beavers with hepatic lesions associated with *E. multilocularis* infection, as evidenced by histology and PCR. Abbreviations: CH: Switzerland; A: Austria; WB: immunoblotting.

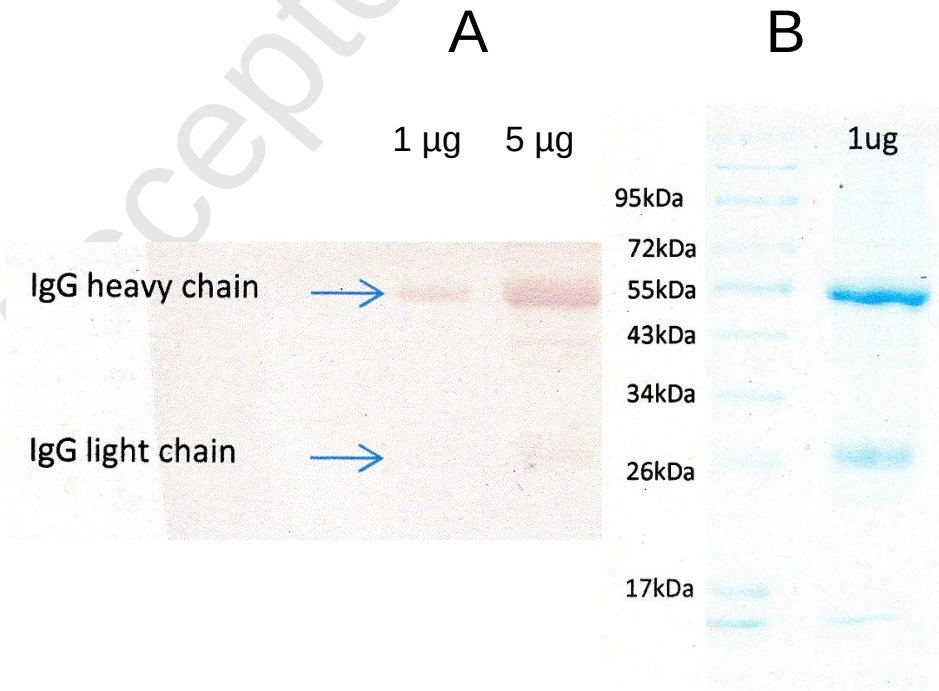
diagnosis	origin	Diagn. no.	Em2_ELISA	Em18_ELISA	WB
AE	CH	12S751	neg	neg	neg
AE	CH	12S752	neg	neg	**pos**
AE	CH	W07/0871	**pos**	neg	**pos**
AE	CH	W08/0973	**pos**	neg	**pos**
AE	CH	W07/4586	**pos**	neg	**pos**
AE	CH	W08/973	neg	neg	**pos**
AE	CH	W07/1152	neg	neg	**pos**
AE	CH	12S759	neg	neg	**pos**
AE	CH	W09/1428	**pos**	neg	**pos**
AE	A	12S751	neg	neg	neg
AE	A	12S752	neg	neg	**pos**
AE	A	12D2967	**pos**	neg	**pos**
AE	A	12D2968	**pos**	neg	**pos**
pos/tot			*6/13*	*0/13*	*11/13*
diagn. sensitivity			**46%**	**0%**	**85%**
"negative"*	Scotland	n = 27	n.d.	n.d.	0/27
"negative"	CH	n = 25	n.d.	n.d.	0/25
"negative"	Austria	n = 4	n.d.	n.d.	0/4
neg/tot					0/60
diagn. specificity					**100%**

*data used to calculate the negative-positive threshold

Legends to Figures

Figure 1: Quality control of the new anti-beaver-IgG-alkaline-phosphate conjugate. (A) Immunoblotting approach with 1 or 5 µg of purified beaver-Ig, SDS-PAGE separated and transferred onto nitrocellulose. Subsequent antibody fragment detection was performed with the new conjugate. (B) Purity status of the purified beaver-IgG used to generate a polyclonal hyperimmune chicken IgY directed against beaver-IgG. The left blue lane shows stained Mr markers and corresponding Mr sizes. The estimated relative molecular mass of the beaver IgG heavy chain is Mr 55 kDa, while that of the light chain appears at approximately Mr 25 kDa.

Figure 1



338 Legends to Figures

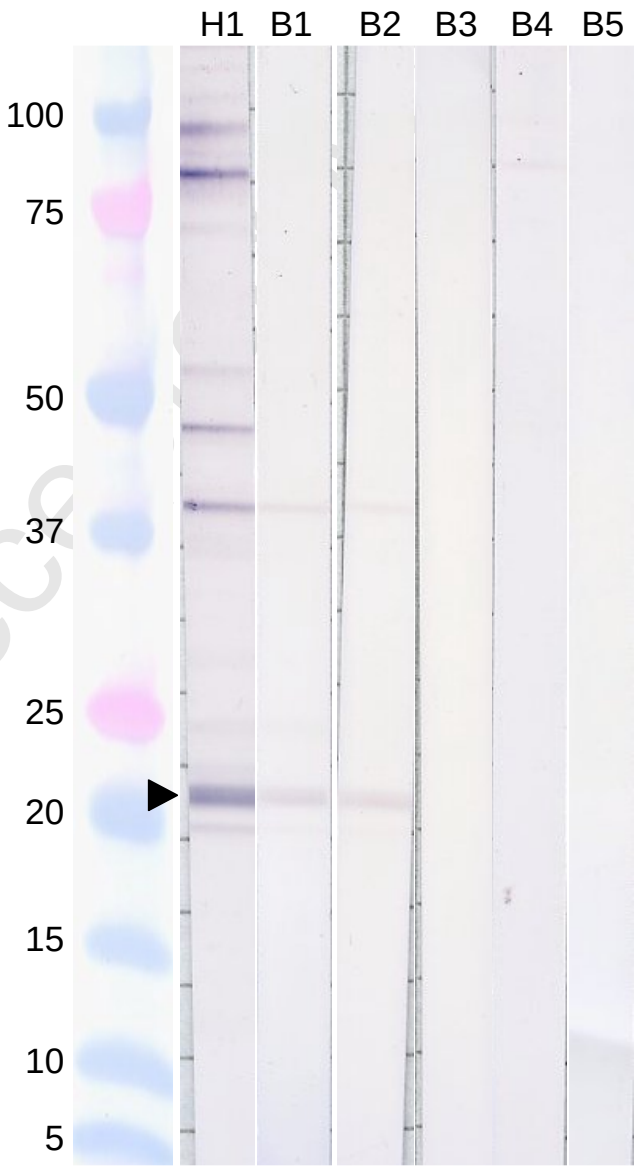
339

340 Figure 2: *E. multilocularis* immunoblot analysis with the following sera: H1 – human AE-patient (positive
341 banding pattern control); B1, B2 - samples from beavers with alveolar echinococcosis (infection
342 confirmed by histology and PCR); B3-B5 – samples from three beavers showing no macroscopic evidence
343 for an infection with *E. multiulocularis* (= negative animals). Arrow points at the diagnostic major band
344 at Mr21. Left lane shows stained Mr markers and corresponding Mr sizes.

345

346 Figure 2

347



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